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(54) Method of synthesizing a peptide containing a non-peptide bond.

(57) This invention features a method for the solid phase synthesis of non-peptide bonds (CH_2-NH) in polypeptide chains. These polypeptides are synthesized by standard procedures and the non-peptide bond synthesized by reacting an amino aldehyde and an amino acid in the presence of $NaCNBH_3$.

This invention also features somatostatin analogs with non peptide bonds.

This invention further features a solid phase method of chemically modifying a peptide. The method involves synthesizing α-N-R and side group N-R analogs of peptides, where R is an alkyl or aryl group, by reacting a carbonyl-containing compound and an amino acid in the presence of $NaCNBH_3$.

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METHOD FOR SYNTHESIZING A PEPTIDE CONTAINING A NON-PEPTIDE

Background of the Invention

This invention relates to the synthesis of polypeptide chains containing non-peptide bonds and to the chemical modification of polypeptide chains.

Normally amino acids within a polypeptide chain are bonded together by a covalent peptide bond of the formula -CO-NH-. A variety of enzymes (proteases) can act on this bond and hydrolyze it to break the polypeptide chain into two or more fragments.

Szelke et al. (1982, Nature 299:555) describe the formation of analogs of angiotensinogen by the chemical modification of peptide bonds within the polypeptide angiotensinogen. The modified bonds have the formula CH_2-NH-, and some of the analogs containing those bonds were found to have increased potency compared to native angiotensinogen. It was hypothesized that this increased potency was due to the inability of proteases to cleave the non-peptide bond. Some of the analogs were synthesized from dipeptides formed in solution by reductive alkylation of an amino acid with an amino aldehyde, using $NaCNBH_3$. The dipeptide was purified by gel filtration and ion-exchange chromatography before completion of the synthesis of the analog.

Summary of the Invention

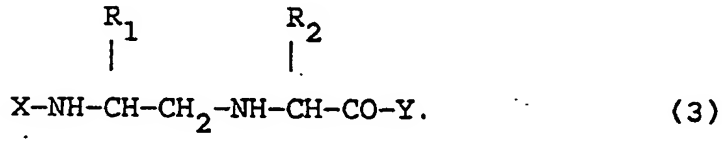
In a first aspect the invention features a method of solid phase synthesis of a polypeptide having a non peptide bond. The method involves providing an amino aldehyde of the formula:

(1)

where X includes a protecting group and R_1 is a side group of an amino acid; providing a complex of the formula:

(2)

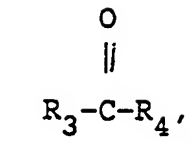
where Y includes a solid phase and R_2 is a side group of an amino acid; and reacting the amino aldehyde with the complex in the presence of sodium cyanoborohydride to form:



(3)

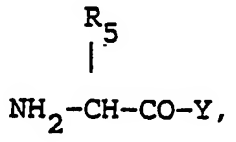
Additional amino acids can then be added to the chain, if desired, and the peptide is then cleaved from the solid-phase to release the polypeptide, which is then purified.

In a second aspect the invention features a method of solid-phase chemical modification of a peptide. The method involves providing a carbonyl containing compound of the formula:



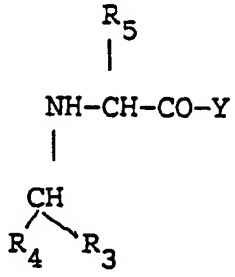
(4)

where R_3 and R_4, independently, include hydrogen; branched or straight chain lower (C_1-C_6) alkyl group, e.g., methyl; or aryl group, e.g., phenyl, p-chloro-phenyl, or naphthyl; providing a complex of the formula:



(5)

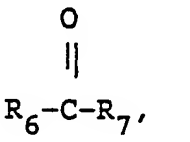
where Y includes a solid phase and R_5 is a side group of an amino acid; and reacting the carbonyl-containing compound with the complex in the presence of sodium cyanoborohydride to form:



(6)

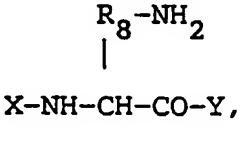
Additional amino acids can then be added to the chain, if desired, and the peptide is then cleaved from the solid-phase to release the peptide, which is then purified. In preferred embodiments, the carbonyl containing compound is formaldehyde ($R_3 = R_4 = H$).

In a third aspect the invention features a method of solid phase chemical modification of a peptide containing amino acid subunits which contain NH_2-containing side groups. The method involves providing a carbonyl containing compound of the formula:



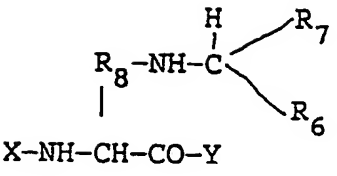
(7)

where R_6 and R_7, independently, include hydrogen; branched or straight chain lower (C_1-C_6) alkyl group, e.g., methyl; or aryl group, e.g., phenyl, p-chloro-phenyl, or naphthyl; providing a complex of the formula:



(8)

where X includes a protecting group, Y includes a solid phase, and R_8-NH_2 is a side group of Lys, ornithine, or diaminobutyric acid; reacting the carbonyl-containing compound with the complex in the presence of sodium cyanoborohydride to form:



; (9)

cleaving off the solid phase to release the peptide; and purifying the peptide. In preferred embodiments, the carbonyl-containing compound is acetone and the R_8-NH_2 side group is a side group of Lys.

In the above formulae (2), (5), and (8), Y can include any number of amino acids which have already been bonded sequentially to a solid phase, e.g., a resin, or Y can consist solely of the solid phase. In other words, the non-peptide bond can link any two amino acids of the peptide, and also can link more than one pair of amino acids in the same peptide. Similarly, X in formulae (1) and (8) can include one or two amino acids, although for purposes of ease of automation, it is preferable that X consist only of a protecting group.

The method can be used to provide increased resistance to proteolytic degradation, and thus longer, half lives in vivo, for any useful synthetic peptides, e.g., human hormones such as LHRH and somatostatin and analogs thereof. The yield, speed, and ease of performance of the method are considerably greater than prior methods using liquid-phase synthesis. In addition, because the method can be used to chemically modify polypeptides in situ, the method provides a simple, fast, and in expensive means for introducing a variety of alkyl and aryl groups into any useful synthetic polypeptides, e.g., hormones and hormone analogs, e.g., somatostatin and LHRH and their therapeutic analogs.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

Drawings

Fig. 1 is a schematic representation of the synthesis of a peptide of the invention featuring a non-peptide bond.

Fig. 2 is a schematic representation of the synthesis of a chemically modified peptide of the invention featuring an α-N-Methyl group.

Fig. 3 is a schematic representation of the synthesis of a chemically modified peptide of the invention featuring an ϵ-isopropyl group.

Structure

Non-peptide bond

By non-peptide bond is meant a -CH_2-NH-moiety between two or more amino acids in a polypeptide chain.

ProtectingGroup

Any suitable standard amino acid protection group can be used. Examples of such protecting groups are FMOC (Fluorenymethyloxycarbonyl) and butyloxycarbonyl (Boc). These groups prevent non specific reaction of the amino acids during synthesis of a polypeptide chain.

Solid phase

The solid phase can be any compound to which an amino acid or polypeptide chain may be reversibly chemically coupled, and upon which synthesis of a polypeptide can be performed. Examples of such solid-phases are resins, e.g., chloromethyl resin and benzhydrylamine-polystyrene resin (Vega Biochemical, Inc.).

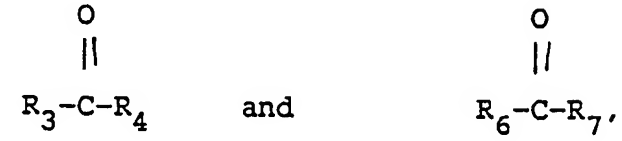
Amino Aldehyde

Amino aldehydes have the general formula:

where X and R_1 are as described above. These compounds generally are synthesized as described by Fehrentz et al. (1983, Synthesis 676).

Carbonyl-containing Compound

Carbonyl-containing compounds have the general formulae:



where R_3, R_4 R_6, and R_7 are as described above. These compounds are commercially available or can be synthesized using conventional techniques.

Somatostatin Analogs

Somatostatin and its analogs are polypeptides with growth hormone-release-inhibiting activity. Some somatostatin analogs have been described in Coy et al. U.S. Patent 4,485,101, hereby incorporated by reference; and Coy et al. U.S.S.N. 775,488, filed September 12, 1985, assigned to the same assignee as the present application and hereby incorporated by reference.

Synthesis

A. Non-peptide analogs of polypeptides

In general, the synthesis of non peptide analogs of polypeptides involves the synthesis of a resin bound protected amino acid or polypeptide chain, and of a protected amino aldehyde, and their reaction in the presence of sodium cyanoborohydride. When the reaction is complete the non-peptide analog can continue to grow, or is cleaved from the resin support and purified by standard procedures.

Example 1

Synthesis of D-Phe-Cys-Tyr-D-Trp-CH_2-NH-Lys-Val-Cys-Thr-NH_2

Referring to the Fig. 1, Boc-D-Trp aldehyde (Boc-D-Trp-CHO, 387 mg, 1.25 mmoles) was prepared (Reaction steps I and II) by the method of Fehrentz et al. (Id.), and dissolved in 5 ml of dry IMF. Briefly, this involved reacting Boc-D-Trp and $CH_3NH(OCH_3)$. HCl in the presence of dicyclohexylcarbodiimide (DCC) and 4-dimethyaminopyridine (DMAP) to form an intermediate, which was then reacted with $LiAlH_4$ in tetrahydrofuran (THF) to form the desired aldehyde.

Boc Lys(Cl-Z)-Val-Cys(MeBzl)-Thr(Bzl)-benzhydrylamine resin (0.5 mmole) was prepared by standard methods using a Beckman 990B automatic peptide synthesizer. The Boc protecting group was removed by treatment with 33% TFA in methylene chloride and the resin TFA salt (TFA NH_2Lys(Cl-Z)-Val-Cys(MeBzl)-Thr(Bzl)-benzyhydrylamine-resin) was suspended in dry dimethylformamide containing 1% of acetic acid (AcOH).

The above aldehyde and resin TFA salt were mixed, and 100 mg (2 mmoles) of sodium cyanoborohydride added (Reaction III). After stirring for I h, the resin mixture was found to be negative to ninhydrin reaction, indicating complete derivatization of the free amino group.

The remaining amino acids of the somatostatin octapeptide (Tyr, Cys, and Phe) were then assembled by standard techniques involving protection steps, carbodiimide couplings and TFA deprotection (Reaction IV).

The free peptide amide was cleaved from the support by treatment with hydrogen-fluoride (HF)/anisole, under standard conditions, and was cyclized by treatment with a slight excess of Iodine (I_2) in 90% acetic acid/water (Reaction V). After evaporation of the solvent, the crude peptide was purified by elution on G-25 in Sephadex™ columns, in 2 M acetic acid, followed by reverse phase partition chromatography on C_{18}-silica using a linear gradient of 10-30% acetonitrile/0.1% trifluoroacetic acid. The purified peptide (the yield was 63.4 mg) was homogeneous by analytical high pressure liquid chromatography (Hplc) and thin layer chromatography (Tlc) in several solvent systems. The material gave the expected ratios after amino acid anlysis of an methanesulfonic acid/tryptamine hydrolysate. The presence of the D-Trp-CH_2NH-Lys pseudodipeptide in the correct ratio was demonstrated by comparison with the elution position of an authentic sample of the dipeptide on the amino acid analyser.

Example 2

Synthesis of D-Phe-Cys-Tyr-D-Trp-Lys-CH_2NH-Val-Cys-Thr-NH_2.

Benzhydrylamine polystyrene resin (1.30 g, 0.5 mmole) in the chloride ion form was placed in the reaction vessel of a Beckman 990B peptide synthesizer programmed to perform the following reaction cycle: (a) methylene chloride; (b) 33% trifluoroacetic acid in methylene chloride (2 times for 1 and 25 min. each); (c) methylene chloride; (d) ethanol: (e) methylene chloride; (f) 10% triethylamine in chloroform.

The neutralized resin was stirred with Boc-O-benzyl-Thr and diisopropylcarbodiimide (1.5 mmole each) in methylene chloride for 1 h and the resulting amino acid resin is then cycled through steps (a) to (f) in the above wash program. The following amino acids (1.5 mmole) were then coupled successively by the same procedure: Boc-s-methylbenzyl-Cys, Boc-Val. The Boc group was then removed by TFA (trifluoroacetic acid) treatment. Boc-Lys (carbenzoxy)-aldehyde (1.25 mmoles), prepared by the method of Fehrentz et al. (id.), was dissolved in 5 ml of dry DMF (dimethylformamide) and added to the resin TFA salt suspension followed by the addition of 100 mg (2 mmoles) of sodium cyanoborohydride. After stirring for 1 h, the resin mixture was found to be negative to ninhydrin reaction (1 min) indicating complete derivatization of the free amino group.

The remaining amino acids, Boc-D-Trp, Boc-tyr, Boc-S-methylbenzyl-Cys, Boc-D-Phe, of the somatostatin octapeptide were then assembled by standard techniques involving carbodiimide couplings and TFA deprotection. After washing and drying, the completed resin weighed 1.87 g.

The resin was mixed with anisole (4 ml) and anhydrous hydrogen fluoride (36 ml) at 0°C and stirred for 45 min; to cleave the peptide from the resin support. Excess hydrogen fluoride was evaporated rapidly under a stream of dry nitrogen and the free peptide precipitated and washed with ether. The crude peptide was then cyclized by dissolving it in 800 ml of 90% acetic acid to which is added I_2 in methanol until a permanent brown color was observed. The solution was stirred for 1 h before removing the solvent in vacuo. The resulting oil was dissolved in a minimum volume of 50% acetic acid and eluted on a column (2.5 × 100 mm) of Sephadex G-25. Fractions containing a major component, as determined by uv (ultraviolet) absorption and thin layer chromatography, were then pooled, evaporated to a small volume and applied to a column (2.5 × 50 cm) of Whatman LRP-1 octadecylsilane (15-20 uM), and eluted with a linear gradient of 10-50% acetonitrile in 0.1% trifluoroacetic acid in water. Fractions were examined by Tlc and analytical Hplc and pooled to give maximum purity. Repeated lyophilization of the solution from water gave 78 mg of the product as a white, fluffy powder.

The product was found to be homgeneous by Hplc and Tlc. Amino acid analysis of an acid hydrolysate confirmed the composition of the octapeptide.

Example 3

Synthesis of D-Phe-Cys-CH_2-NH-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2.

This peptide was assembled on benzyhdrylamine resin according to the conditions described in Example 2, using Boc-O-benzyl-Thr, Boc S-methylbenzyl-Cys, Boc-Val, Boc Lys, N-benzyloxycarbonyl-Lys, Boc-D-Trp and 2-bromocarbenzoxy-Tyr. Boc-Cys(methylbenzyl)-aldehyde was then added to the resin along with $NaCNBH_3$, as in Example 2. The remaining amino acid, Boc-tert-butyloxycarbonyl-D-Phe, was then added as in Example 2. The final resin weighed 1.84 g.

The resin was subjected to hydrogen fluoride cleavage and I_2 cyclization as described in Example 2. The lyophilized product weighed 89 mg and was found to be homogeneous by Hplc and Tlc. Amino acid analysis of an acid hydrolysate confirmed the composition of the octapeptide.

The above reductive amination method is particularly applicable to peptides containing Trp and Cys residues, and is also compatible with Bzl and Cbz-type side-chain protecting groups.

B. α-N-R analogsof polypeptides

In general, the synthesis of α-N-R analogs of polypeptides, where R is an alkyl or aryl group, involves the synthesis of a resin-bound protected amino acid or polypeptide chain, and of a carbonyl-containing compound, and their reaction in the presence of sodium cyanoborohydride. The net effect of the reaction is to convert the carbonyl group

(- $\overset{O}{\overset{\|}{C}}$ -) to a CH group bonded to the nitrogen atom of the α-amino group of the resin-bound amino acid or polypeptide. For example, if the carbonyl compound is formaldehyde

(H $\overset{O}{\overset{\|}{C}}$ H), the reaction produces an α-N-methyl moiety. When the sodium cyanoborohydride reaction is complete, the modified peptide can continue to grow, or is cleaved from the resin support and purified by standard procedures.

Example 1

Synthesis of The LHRH Agonist pGlu-His-Trp-Ser-Tyr-D-Ala-N-Me-Leu-Arg-Pro-Gly-NH_2

Referring to Fig. 2, TFA. NH_2-Leu-Arg (Tos)-Pro-Gly-benzhydrylamine resin (0.5 mmole) was prepared by standard methods using a Beckman 990B automatic peptide synthesizer. The resin TFA salt was then mixed with 2 ml formaldehyde (37% formalin), and 1.5 mmoles of sodium cyanoborohydride in DMF (dimethylformamide)/1% ACOH (acetic acid) added. The resin mixture was stirred until it was negative to ninhydrin reaction, indicating complete derivatization of the free amino group to form an N-Methyl amino group.

The remaining amino acids of the polypeptide (D-Ala, Tyr, Ser, Trp, His, and pGlu) were then assembled by standard techniques involving protection steps, carbodiimide couplings, and TFA deprotection.

The free peptide amide was then cleaved from the support by treatment with HF/anisole and purified under standard conditions to yield the desired polypeptide.

C. Side group N-R analogs of polypeptides

In general, the synthesis of side group N-R analogs of polypeptides, where R is an alkyl or aryl group, involves reacting a resin-bound protected amino acid featuring a side chain containing a free amino group, or a resin-bound polypeptide chain containing such an amino acid subunit, with a carbonyl-containing compound in the presence of sodium cyanoborohydride. Amino acids featuring a side chain containing a free amino group include Lys, ornithine, and diaminobutyric acid. The net effect of the reaction is to convert the carbonyl group

(- $\overset{O}{\overset{\|}{C}}$ -) into a CH group bonded to the nitrogen of the sidechain free amino group of the resin-bound amino

acid or polypeptide. For example, if the carbonyl compound is acetone

O

($CH_3C\ C\ H_3$) and the amino acid with the free amino-containing side chain is Lys (side chain = $-(CH_2)_3-CH_2NH_2$), the reaction produces an ϵ-N-isopropyl moiety. When the sodium cyanoborohydride reaction is complete, the modified peptide is cleaved from the resin support and purified by standard procedures.

The above-described synthesis can be used to prepare LHRH antagonists, as described below.

Example 1

Synthesis of The LHRH Antagonist Ac-D-Nal-D-Phe-D-Phe-Ser-Tyr-D-Lys(iPr)-Phe-Lys (iPr)-Pro-Ala-NH_2

Referring to Fig. 3,
Ac-D-Nal-D-Phe-D-Phe-Ser(Bzl)-Tyr(Bzl)-D-Lys(FMOC)-Phe-D-Lys(FMOC)-Pro-D-Ala-benzhydrylamin e (0.25 mmole) resin was prepared by standard methods in a Beckman 990B automatic peptide synthesizer using 33% TFA for removal of the O-BOC protecting groups. The ϵ-FMOC protecting groups on the Lys residues are completely stable to these acidic conditions, and to subsequent neutralization steps with 10% triethylamine in chloroform. The resin was then treated with 50ml of a 50% solution of piperidine in DMF (dimethylformamide) for about 12h to remove the FMOC protecting group from the Lys residues.

To react the free ϵ-amino group of the Lys residues, the resin was mixed with 5ml of acetone, and 1 mmole of sodium cyanoborohydride in DMF/1% acetic acid added. The resin mixture was then stirred until it was negative to ninhydrin reaction (about 3h); the negative ninhydrin reaction indicated that the free ϵ-amino group had been converted to N-isopropyl amino groups.

The resin was then cleaved from the support by treatment with HF/anisole and purified under standard conditions to yield the desired polypeptide.

Ac-D-Nal-D-Phe-D-Phe-Ser-Tyr-D-Lys(iPr)-Phe-Arg-Pro-D-Ala-amide is prepared in analagous fashion using appropriate modifications of the above-described procedure.

Use

The method of the invention can be used to modify any peptides of therapeutic or veterinary interest, e.g., hormones such as LHRH, TRH, and somatostatin, and analogs thereof. Such modifications can increase the chemical stability and potency of the peptides. In addition, the introduction of N-alkyl or aryl groups will minimize undesirable side effects, e.g., skin irritation, which are often present when the unalkylated or unarylated peptides are administered to human patients. Furthermore, the invention permits the addition of side groups (e.g., isopropyl) to amino acids in an inexpensive way, compared to methods in which the expensive pre-derivatized amino acid itself is employed.

Other embodiments are within the following claims.

Claims

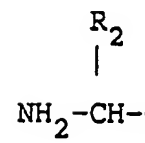
1. A method of solid-phase synthesis of a polypeptide having a non-peptide bond, comprising the steps of

(a) providing an amino aldehyde of the formula

$$X-NH-\underset{\displaystyle R_1}{\underset{|}{CH}}-COH,$$

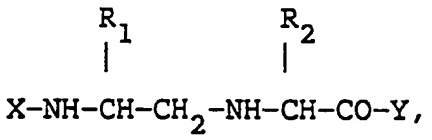
where X comprises a protecting group and R_1 is a side group of an amino acid,

(b) providing a complex of the formula



where Y comprises a solid phase and R_2 is a side group of an amino acid, and

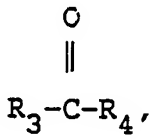
(c) reacting said amino aldehyde with said complex in the presence of sodium cyanoborohydride to form



2. A method as claimed in claim 1 in which the said amino aldehyde is Trp aldehyde, Lys aldehyde or Cys aldehyde.

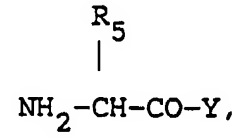
3. A method of solid-phase chemical modification of a peptide, comprising the steps of

a) providing a carbonyl-containing compound of the formula



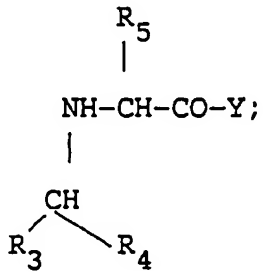
where R_3 and R_4, independently, comprise hydrogen, branched or straight chain lower alkyl group, or aryl group;

(b) providing a complex of the formula



where Y comprises a solid phase and R_5 is a side group of an amino acid; and

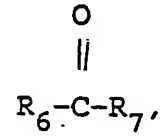
(c) reacting said carbonyl-containing compound with said complex in the presence of sodium cyanoborohydride to form



4. A method as claimed in claim 3 in which the said carbonyl-containing compound is formaldehyde.

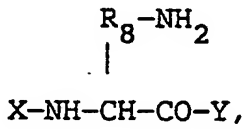
5. A method of solid-phase chemical modification of a peptide, comprising the steps of

(a) providing a carbonyl-containing compound of the formula



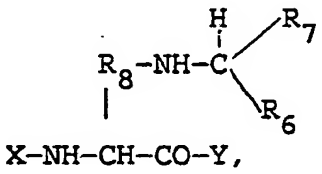
where R_6 and R_7, independently, comprise hydrogen, branched or straight chain lower alkyl group, or aryl group;

(b) providing a complex of the formula



where X comprises a protecting group, Y comprises a solid phase, and R_8-NH_2 is a side group of Lys, ornithine, or diaminobutyric acid;

(c) reacting said carbonyl-containing compound with said complex in the presence of sodium cyanoborohydride to form



(d) cleaving off said solid phase to release said peptide; and

(e) purifying said peptide.

6. A method as claimed in claim 5 in which the said carbonyl-containing compound is acetone.

7. A method as claimed in claim 4 or claim 5 in which the R_8-NH_2 side group is a side group of Lys.

8. A method as claimed in any one of claims 1 to 7 in which the said solid phase is a resin.

9. A method as claimed in claim 8 in which the said resin is chloromethyl resin or benzhydrylamine-polystyrene resin.

10. A method as claimed in any one of claims 1 to 9 further comprising the step of cyclizing said polypeptide.

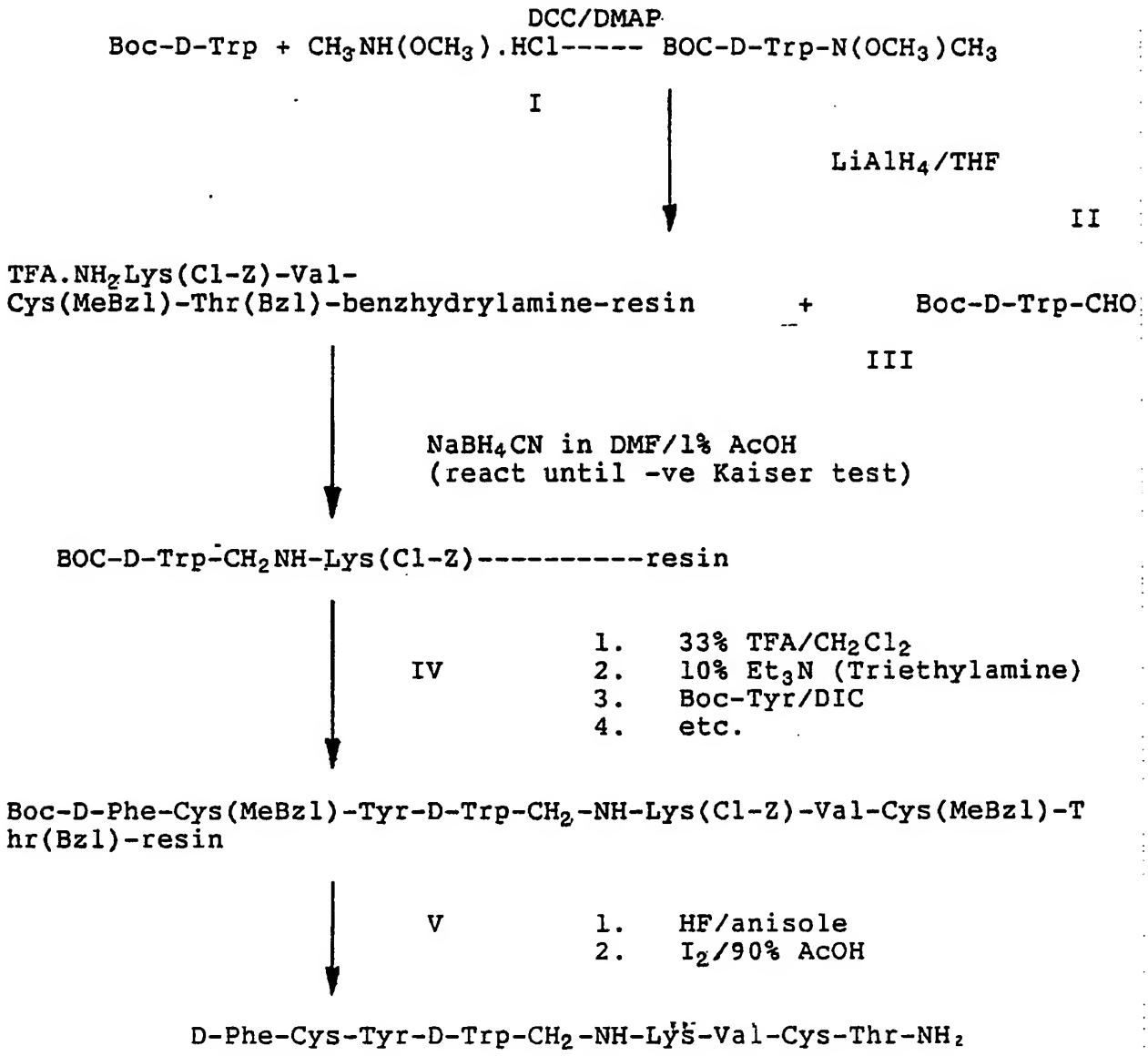
11. A peptide prepared by a method as claimed in any one of the preceding claims.

12. A peptide in which two amino acids are bonded by a non-peptide bond introduced by a method as claimed in any one of the preceding claims.

13. A peptide as claimed in claim 11 or claim 12 the said peptide being a somatostatin analog.

14. A peptide as claimed in claim 11 in which the said peptide comprises an N-methyl leucine group.

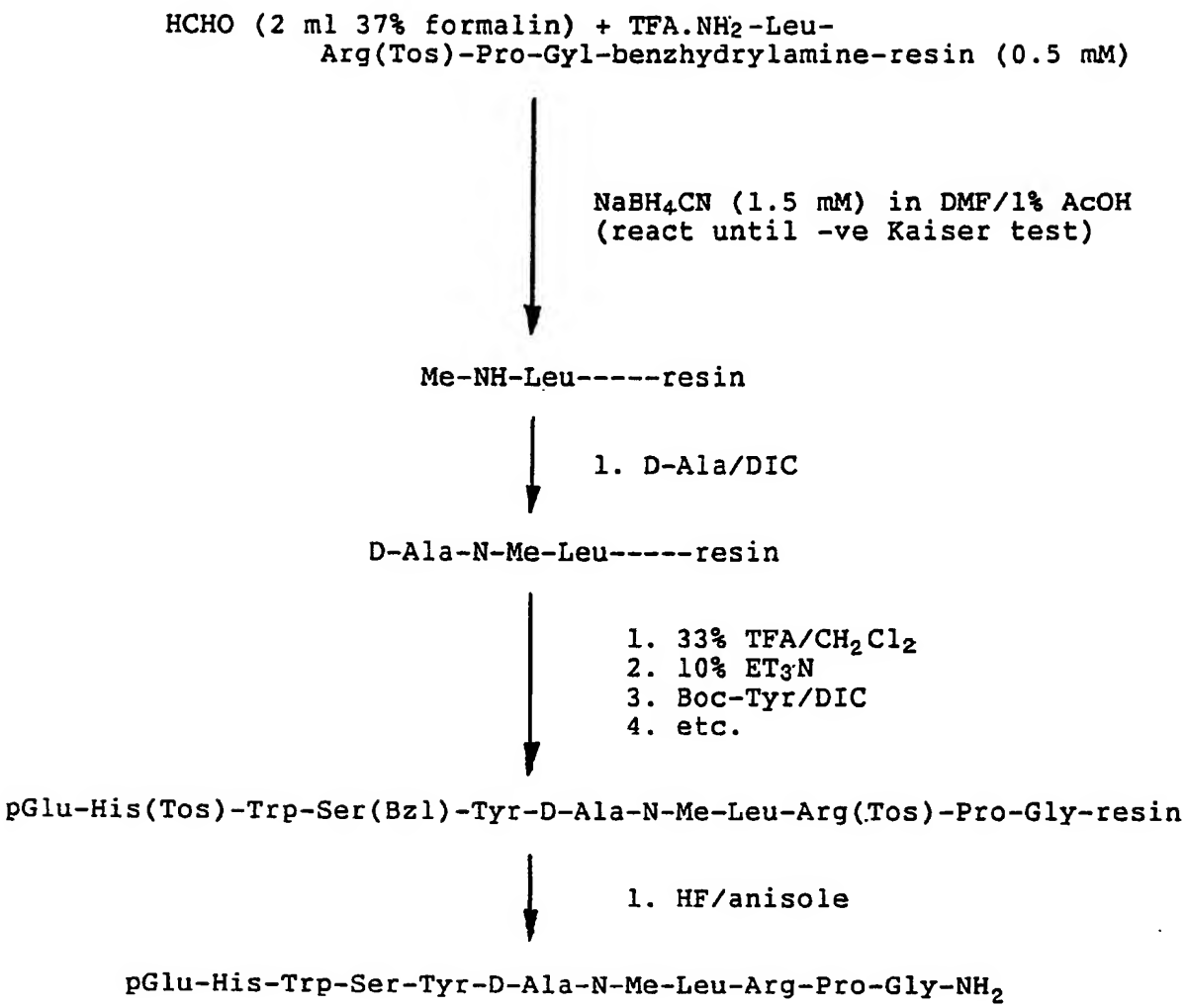
15. A peptide as claimed in claim 11 in which the said polypeptide comprises an ϵ-isopropyl lysine group.



Abbreviations:

TFA:	trifluoroacetic acid
THF:	tetrahydrofuran
MeBzl:	methybenzyl
Bzl:	benzyl
Cl-Z:	4-chlorocabenzoxy
DMAP:	4-dimethyaminopyridine
DCC:	dicyclohexylcarbodiimide
DIC:	diisopropylcarbodiimide
HF:	hydrogenfluoride
I-V:	Reaction steps

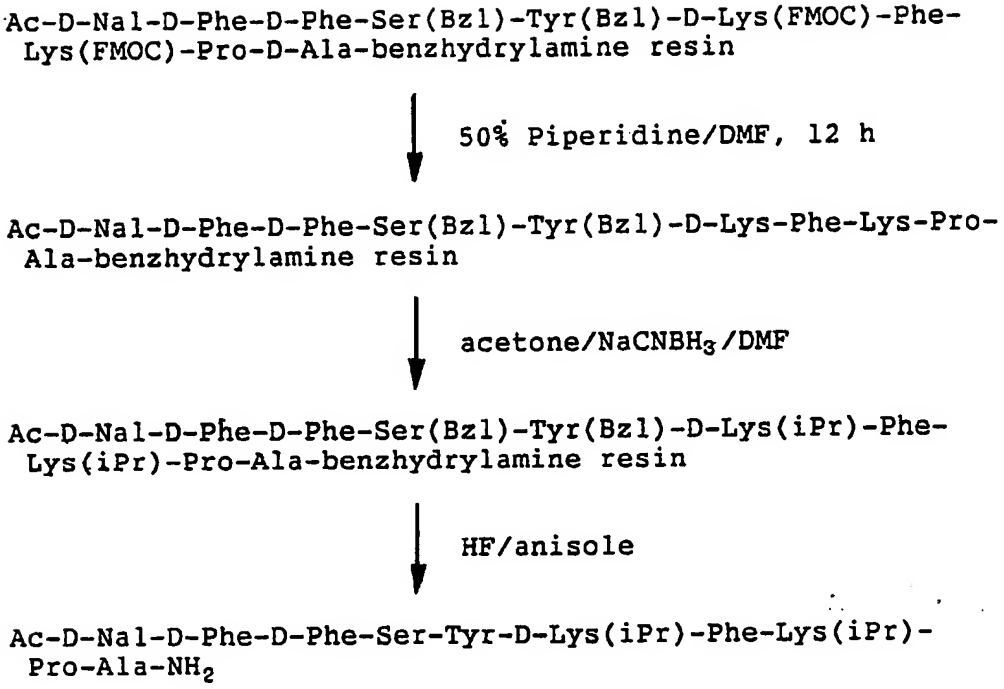
FIG. 1



Abbreviations:

TFA:	trifluoroacetic acid
DIC:	diisopropylcarbodiimide
HF:	hydrogenfluoride
Tos:	tosyl
Bzl:	benzyl

FIG. 2



Abbreviations:

FMOC:	fluorenylmethyloxycarbonyl
iPr:	epsilon isopropyl
Bzl:	benzyl

FIG. 3

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(57) Abstract

A linear peptide which is an analog of a naturally occurring, biologically active bombesin having an active site and a binding site responsible for binding of bombesin to a receptor on a target cell, cleavage of a peptide bond in the active site of the naturally occurring peptide being unnecessary for *in vivo* biological activity, the analog having a non-peptide bond instead of a peptide bond between an amino acid of the active site and an adjacent amino acid, and having the same binding site as the naturally occurring peptide, so that the analog is capable of acting as a competitive inhibitor of naturally occurring bombesin by binding to the receptor and, by virtue of the non-peptide bond, failing to exhibit the *in vivo* activity of naturally occurring bombesin.

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Therapeutic Peptides

Background of the Invention

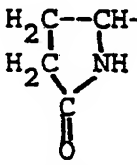
This invention relates to therapeutic peptides useful, e.g., in cancer therapy.

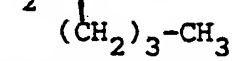
The amphibian peptide bombesin, pGlu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2 (Anastasi et al., Experientia 27:166-167 (1971)), is closely related to the mammalian gastrin-releasing peptides (GRP), e.g., the porcine GRP, H_2N-Ala-Pro-Val-Ser-Val-Gly-Gly-Gly-Thr-Val-Leu-Ala-Lys-Met-Tyr-Pro-Arg-Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met-(NH_2) (McDonald et al., Biochem. Biophys. Res. Commun. 90:227-233 (1979)) and human GRP, H_2N-Val-Pro-Leu-Pro-Ala-Gly-Gly-Gly-Thr-Val-Leu-Thr-Lys-Met-Tyr-Pro-Arg-Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met-NH_2. Bombesin has been found to be an autocrine or paracrine mitotic factor for a number of human cancer cell lines, including small-cell lung carcinoma (SCLC) (Haveman et al., eds. Recent Results in Cancer Research - Peptide Hormones in Lung Cancer, Springer-Verlag, New York:1986). A number of these cancers are known to secrete peptide hormones related to GRP or bombesin. Consequently, antagonists to bombesin have been proposed as agents for the treatment of these cancers.

Cuttitta et al. demonstrated that a specific monoclonal antibody to bombesin inhibited in vivo the growth of a human small-cell lung cancer cell line xenografted to nude mice (Cuttitta et al., Cancer Survey 4:707-727 (1985)). In 3T3 murine fibroblasts which are responsive to the mitotic effect of bombesin, Zachary and Rozengurt observed that a substance P antagonist (Spantide) acted as a bombesin antagonist (Zachary et al., Proc. Natl. Acad. Sci. (USA), 82:7616-7620 (1985)).

Heinz-Erian et al. replaced His at position 12 in bombesin with D-Phe and observed bombesin antagonist activity in dispersed acini from guinea pig pancreas (Heinz-Erian et al., Am. J. of Physiol. 252:G439-G442 (1987)). Rivier reported on work directed toward restricting the conformational freedom of the bioactive C-terminal decapeptide of bombesin by incorporating intramolecular disulfide bridges; however, Rivier mentioned that, so far, bombesin analogs with this modification fail to exhibit any antagonist activity (Rivier et al., "Competitive Antagonists of Peptide Hormones," in Abstracts of the International Symposium on Bombesin-Like Peptides in Health and Disease, Rome (October, 1987).

Abbreviations (uncommon):

pGlu =  (pyroglutamate);

Nle =  (norleucine)

Pal = 3-pyridyl-alanine

Nal = naphthylalanine

Summary of the Invention

In general, the invention features a linear (i.e., non-cyclic) peptide which is an analog of a naturally occurring, biologically active bombesin having an active site and a binding site responsible for the binding of bombesin to a receptor on a target cell, cleavage of a peptide bond in the active site of naturally occurring bombesin being unnecessary for in vivo biological activity, the analog having a non-peptide bond instead of a peptide bond between an amino acid of the active site and an adjacent amino acid, the analog being capable of binding to the receptor, so that the analog is capable of acting as a competitive inhibitor of naturally occurring bombesin by binding to the receptor and, by virtue of

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(54) **Method of synthesizing a peptide containing a non-peptide bond.**

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(56) References cited:

JOURNAL OF MEDICINAL CHEMISTRY, vol. 28, no. 12, December 1985, page 1874, American Chemical Society, Washington, DC, US; J. MARTINEZ et al.: "Synthesis and biological activities of some pseudo-peptide analogues of tetragastrin: The importance of the peptide backbone"

THE JOURNAL OF ORGANIC CHEMISTRY, vol. 37, no. 10, 19th May 1972, pages 1673-1674, The American Chemical Society, Washington, US; R.F. BORCH et al.: "A new method for the methylation of amines"

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CHEMICAL ABSTRACTS, vol. 101, no. 3, 16th July 1984, page 643, abstract no. 23909w, Columbus, Ohio, US; Y. OHFUNE et al.: "An efficient one-step reductive N-monoalkylation of a-amine acids", & CHEM. LETT. 1984, (3), 441-4

CHEMICAL ABSTRACTS, vol. 108, no. 15, 11th April 1988, page 803, abstract no. 132280z, Columbus, Ohio, US; Y. SASAKI et al.: "Solid phase synthesis of peptides containing the Ch2NH peptide bond isostere", & PEPTIDES (FAYETTEVILLE, N. Y.) 1987, 8(1), 119-21

TETRAHEDRON, vol. 44, no. 3, 1988, pages 835-841, Pergamon Press, Oxford, GB; D.H. COY et al.: "Solid phase reductive alkylation techniques in analogue peptide bond and sidechain modification"

Y Yasati and D H Coy, Peptides, 8:119-121(1986)

Description

Background of The Invention

This invention relates to the synthesis of polypeptide chains containing non-peptide bonds and to the chemical modification of polypeptide chains.

Normally amino acids within a polypeptide chain are bonded together by a covalent peptide bond of the formula -CO-NH-. A variety of enzymes (proteases) can act on this bond and hydrolyze it to break the polypeptide chain into two or more fragments.

Szelke et al. (1982, Nature 299:555) describe the formation of analogs of angiotensinogen by the chemical modification of peptide bonds within the polypeptide angiotensinogen. The modified bonds have the formula CH_2-NH-, and some of the analogs containing those bonds were found to have increased potency compared to native angiotensinogen. It was hypothesized that this increased potency was due to the inability of proteases to cleave the non-peptide bond. Some of the analogs were synthesized from dipeptides formed in solution by reductive alkylation of an amino acid with an amino aldehyde, using $NaCNBH_3$. The dipeptide was purified by gel filtration and ion-exchange chromatography before completion of the synthesis of the analog.

Summary of the Invention

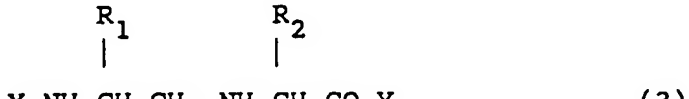
In a first aspect the invention features a method of solid phase synthesis of a polypeptide having a non peptide bond. The method involves providing an amino aldehyde of the formula:

(1)

where X includes a protecting group and R_1 is a side group of an amino acid; providing a complex of the formula:

(2)

where Y includes a solid phase and R_2 is a side group of an amino acid; and reacting the amino aldehyde with the complex in the presence of sodium cyanoborohydride to form:



(3)

Additional amino acids can then be added to the chain, if desired, and the peptide is then cleaved from the solid-phase to release the polypeptide, which is then purified.

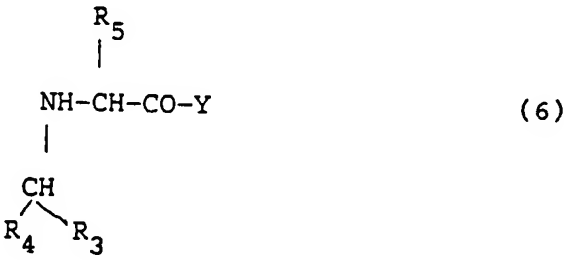
In a second aspect the invention features a method of solid-phase chemical modification of a peptide. The method involves providing a carbonyl - containing compound of the formula:

(4)

where R_3 and R_4, independently, include hydrogen; branched or straight chain lower (C_1-C_6) alkyl group, e.g., methyl; or aryl group, e.g., phenyl, p-chloro-phenyl, or naphthyl; providing a complex of the formula:

(5)

where Y includes a solid phase and R_5 is a side group of an amino acid; and reacting the carbonyl-containing compound with the complex in the presence of sodium cyanoborohydride to form:



(6)

Additional amino acids can then be added to the chain, if desired, and the peptide is then cleaved from the solid-phase to release the peptide, which is then purified. In preferred embodiments, the carbonyl containing compound is formaldehyde ($R_3 = R_4 = H$).

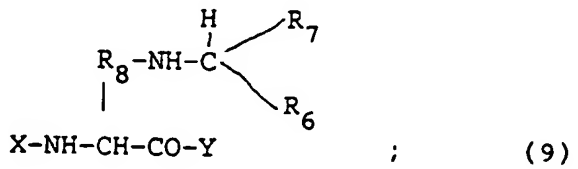
In a third aspect the invention features a method of solid phase chemical modification of a peptide containing amino acid subunits which contain NH_2-containing side groups. The method involves providing a carbonyl containing compound of the formula:

(7)

where R_6 and R_7, independently, include hydrogen; branched or straight chain lower (C_1-C_6) alkyl group, e.g., methyl; or aryl group, e.g., phenyl, p-chloro-phenyl, or naphthyl; providing a complex of the formula:

(8)

where X includes a protecting group, Y includes a solid phase, and R_8-NH_2 is a side group of Lys, ornithine, or diaminobutyric acid; reacting the carbonyl-containing compound with the complex in the presence of sodium cyanoborohydride to form:



(9)

cleaving off the solid phase to release the peptide; and purifying the peptide. In preferred embodiments, the carbonyl-containing compound is acetone and the R_8-NH_2 side group is a side group of Lys.

In the above formulae (2), (5), and (8), Y can include any number of amino acids which have already been bonded sequentially to a solid phase, e.g., a resin, or Y can consist solely of the solid phase. In other words, the non-peptide bond can link any two amino acids of the peptide, and also can link more than one pair of amino acids in the same peptide. Similarly, X in formulae (1) and (8) can include one or two amino acids, although for purposes of ease of automation, it is preferable that X consist only of a protecting group.

The method can be used to provide increased resistance to proteolytic degradation, and thus longer, half lives in vivo, for any useful synthetic peptides, e.g., human hormones such as LHRH and somatostatin and analogs thereof. The yield, speed, and ease of performance of the method are considerably greater than prior methods using liquid-phase synthesis. In addition, because the method can be used to chemically modify polypeptides in situ, the method provides a simple, fast, and in expensive means for introducing a variety of alkyl and aryl groups into any useful synthetic polypeptides, e.g., hormones and hormone analogs, e.g., somatostatin and LHRH and their therapeutic analogs.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

Drawings

Fig. 1 is a schematic representation of the synthesis of a peptide of the invention featuring a non-peptide bond.

Fig. 2 is a schematic representation of the synthesis of a chemically modified peptide of the invention featuring an $^{\alpha}$-N-Methyl group.

Fig. 3 is a schematic representation of the synthesis of a chemically modified peptide of the invention featuring an $^{\alpha}$-isopropyl group.

Structure

Non-peptide bond

By non-peptide bond is meant a -CH_2-NH-moiety between two or more amino acids in a polypeptide chain.

Protecting Group

Any suitable standard amino acid protection group can be used. Examples of such protecting groups are FMOC (Fluorenymethyloxycarbonyl) and butyloxycarbonyl (Boc). These groups prevent non specific reaction of the amino acids during synthesis of a polypeptide chain.

Solid phase

The solid phase can be any compound to which an amino acid or polypeptide chain may be reversibly chemically coupled, and upon which synthesis of a polypeptide can be performed. Examples of such solid-phases are resins, e.g., chloromethyl resin and benzhydrylamine-polystyrene resin (Vega Biochemical, Inc.).

Amino Aldehyde

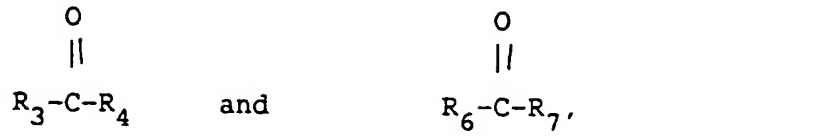
Amino aldehydes have the general formula:

$$X\text{-}NH\text{-}\underset{\displaystyle R_1}{\underset{|}{CH}}\text{-}CHO,$$

where X and R_1 are as described above. These compounds generally are synthesized as described by Fehrentz et al. (1983, Synthesis 676).

Carbonyl-containing Compound

Carbonyl-containing compounds have the general formulae:



where R_3, R_4 R_6, and R_7 are as described above. These compounds are commercially available or can be synthesized using conventional techniques.

Somatostatin Analogs

Somatostatin and its analogs are polypeptides with growth hormone-release-inhibiting activity. Some somatostatin analogs have been described in Coy et al. U.S. Patent 4,485,101, hereby incorporated by reference; and Coy et al. U.S.S.N. 775,488, filed September 12, 1985, assigned to the same assignee as the present application and hereby incorporated by reference.

Synthesis

A. Non-peptide analogs of polypeptides

In general, the synthesis of non peptide analogs of polypeptides involves the synthesis of a resin bound protected amino acid or polypeptide chain, and of a protected amino aldehyde, and their reaction in the presence of sodium cyanoborohydride. When the reaction is complete the non-peptide analog can continue to grow, or is cleaved from the resin support and purified by standard procedures.

Example 1

Synthesis of

D-Phe-Cys-Tyr-D-Trp-CH_2-NH-Lys-Val-Cys-Thr-NH_2

Referring to the Fig. 1, Boc-D-Trp aldehyde (Boc-D-Trp-CHO, 387 mg, 1.25 mmoles) was prepared (Reaction steps I and II) by the method of Fehrentz et al. (id.), and dissolved in 5 ml of dry IMF. Briefly, this involved reacting Boc-D-Trp and $CH_3NH(OCH_3)$. HCl in the presence of dicyclohexylcarbodiimide (DCC) and 4-dimethyaminopyridine (DMAP) to form an intermediate, which was then reacted with $LiAlH_4$ in tetrahydrofuran (THF) to form the desired aldehyde.

Boc-Lys(Cl-Z)-Val-Cys(MeBzl)-Thr(Bzl)-benzhydrylamine resin (0.5 mmole) was prepared by standard methods using a Beckman 990B automatic peptide synthesizer. The Boc protecting group was removed by treatment with 33% TFA in methylene chloride and the resin TFA salt (TFA NH_2Lys(Cl-Z)-Val-Cys(MeBzl)-Thr(Bzl)-benzyhydrylamine-resin) was suspended in dry dimethylformamide containing 1% of acetic acid (AcOH).

The above aldehyde and resin TFA salt were mixed, and 100 mg (2 mmoles) of sodium cyanoborohydride added (Reaction III). After stirring for 1 h, the resin mixture was found to be negative to ninhydrin reaction, indicating complete derivatization of the free amino group.

The remaining amino acids of the somatostatin octapeptide (Tyr, Cys, and Phe) were then assembled by standard techniques involving protection steps, carbodiimide couplings and TFA deprotection (Reaction IV).

The free peptide amide was cleaved from the support by treatment with hydrogen-fluoride (HF)/anisole, under standard conditions, and was cyclized by treatment with a slight excess of Iodine (I_2) in 90% acetic acid/water (Reaction V). After evaporation of the solvent, the crude peptide was purified by elution on G-25

in Sephadex™ columns, in 2 M acetic acid, followed by reverse phase partition chromatography on C_{18}-silica using a linear gradient of 10-30% acetonitrile/0.1% trifluoroacetic acid. The purified peptide (the yield was 63.4 mg) was homogeneous by analytical high pressure liquid chromatography (Hplc) and thin layer chromatography (Tlc) in several solvent systems. The material gave the expected ratios after amino acid anlysis of an methanesulfonic acid/tryptamine hydrolysate. The presence of the D-Trp-CH_2NH-Lys pseudodipeptide in the correct ratio was demonstrated by comparison with the elution position of an authentic sample of the dipeptide on the amino acid analyser.

Example 2

Synthesis of

D-Phe-Cys-Tyr-D-Trp-Lys-CH_2NH- Val-Cys-Thr-NH_2.

Benzhydrylamine-polystyrene resin (1.30 g, 0.5 mmole) in the chloride ion form was placed in the reaction vessel of a Beckman 990B peptide synthesizer programmed to perform the following reaction cycle: (a) methylene chloride; (b) 33% trifluoroacetic acid in methylene chloride (2 times for 1 and 25 min. each); (c) methylene chloride; (d) ethanol: (e) methylene chloride; (f) 10% triethylamine in chloroform.

The neutralized resin was stirred with Boc-O-benzyl-Thr and diisopropylcarbodiimide (1.5 mmole each) in methylene chloride for 1 h and the resulting amino acid resin is then cycled through steps (a) to (f) in the above wash program. The following amino acids (1.5 mmole) were then coupled successively by the same procedure: Boc-s-methylbenzyl-Cys, Boc-Val. The Boc group was then removed by TFA (trifluoroacetic acid) treatment. Boc-Lys (carbenzoxy)-aldehyde (1.25 mmoles), prepared by the method of Fehrentz et al. (id.), was dissolved in 5 ml of dry DMF (dimethylformamide) and added to the resin TFA salt suspension followed by the addition of 100 mg (2 mmoles) of sodium cyanoborohydride. After stirring for 1 h, the resin mixture was found to be negative to ninhydrin reaction (1 min) indicating complete derivatization of the free amino group.

The remaining amino acids, Boc-D-Trp, Boc-tyr, Boc-S-methylbenzyl-Cys, Boc-D-Phe, of the somatostatin octapeptide were then assembled by standard techniques involving carbodiimide couplings and TFA deprotection. After washing and drying, the completed resin weighed 1.87 g.

The resin was mixed with anisole (4 ml) and anhydrous hydrogen fluoride (36 ml) at 0 °C and stirred for 45 min; to cleave the peptide from the resin support. Excess hydrogen fluoride was evaporated rapidly under a stream of dry nitrogen and the free peptide precipitated and washed with ether. The crude peptide was then cyclized by dissolving it in 800 ml of 90% acetic acid to which is added I_2 in methanol until a permanent brown color was observed. The solution was stirred for 1 h before removing the solvent in vacuo. The resulting oil was dissolved in a minimum volume of 50% acetic acid and eluted on a column (2.5 X 100 mm) of Sephadex G-25. Fractions containing a major component, as determined by uv (ultraviolet) absorption and thin layer chromatography, were then pooled, evaporated to a small volume and applied to a column (2.5 X 50 cm) of Whatman LRP-1 octadecylsilane (15-20 uM), and eluted with a linear gradient of 10-50% acetonitrile in 0.1% trifluoroacetic acid in water. Fractions were examined by Tlc and analytical Hplc and pooled to give maximum purity. Repeated lyophilization of the solution from water gave 78 mg of the product as a white, fluffy powder.

The product was found to be homogeneous by Hplc and Tlc. Amino acid analysis of an acid hydrolysate confirmed the composition of the octapeptide.

Example 3

Synthesis of

D-Phe-Cys-CH_2-NH-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2.

This peptide was assembled on benzyhdrylamine resin according to the conditions described in Example 2, using Boc-O-benzyl-Thr, Boc S-methylbenzyl-Cys, Boc-Val, Boc Lys, N-benzyloxycarbonyl-Lys, Boc-D-Trp and 2-bromocarbenzoxy-Tyr. Boc-Cys(methylbenzyl)-aldehyde was then added to the resin along with $NaCNBH_3$, as in Example 2. The remaining amino acid, Boc-tert-butyloxycarbonyl-D-Phe, was then added as in Example 2. The final resin weighed 1.84 g.

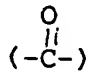
The resin was subjected to hydrogen fluoride cleavage and I_2 cyclization as described in Example 2. The lyophilized product weighed 89 mg and was found to be homogeneous by Hplc and Tlc. Amino acid

analysis of an acid hydrolysate confirmed the composition of the octapeptide.

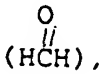
The above reductive amination method is particularly applicable to peptides containing Trp and Cys residues, and is also compatible with Bzl and Cbz-type side-chain protecting groups.

B. $^{\alpha}$-N-R analogs of polypeptides

In general, the synthesis of $^{\alpha}$-N-R analogs of polypeptides, where R is an alkyl or aryl group, involves the synthesis of a resin-bound protected amino acid or polypeptide chain, and of a carbonyl-containing compound, and their reaction in the presence of sodium cyanoborohydride. The net effect of the reaction is to convert the carbonyl group



to a CH group bonded to the nitrogen atom of the $^{\alpha}$-amino group of the resin-bound amino acid or polypeptide. For example, if the carbonyl compound is formaldehyde



the reaction produces an $^{\alpha}$-N-methyl moiety. When the sodium cyanoborohydride reaction is complete, the modified peptide can continue to grow, or is cleaved from the resin support and purified by standard procedures.

Example 1

Synthesis of The LHRH Agonist

pGlu-His-Trp-Ser-Tyr-D-Ala-N-Me-Leu-Arg-Pro-Gly-NH_2

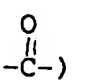
Referring to Fig. 2, TFA. NH_2-Leu-Arg (Tos)-Pro-Gly-benzhydrylamine resin (0.5 mmole) was prepared by standard methods using a Beckman 990B automatic peptide synthesizer. The resin TFA salt was then mixed with 2 ml formaldehyde (37% formalin), and 1.5 mmoles of sodium cyanoborohydride in DMF (dimethylformamide)/1% ACOH (acetic acid) added. The resin mixture was stirred until it was negative to ninhydrin reaction, indicating complete derivatization of the free amino group to form an N-Methyl amino group.

The remaining amino acids of the polypeptide (D-Ala, Tyr, Ser, Trp, His, and pGlu) were then assembled by standard techniques involving protection steps, carbodiimide couplings, and TFA deprotection.

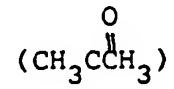
The free peptide amide was then cleaved from the support by treatment with HF/anisole and purified under standard conditions to yield the desired polypeptide.

C. Side group N-R analogs of polypeptides

In general, the synthesis of side group N-R analogs of polypeptides, where R is an alkyl or aryl group, involves reacting a resin-bound protected amino acid featuring a side chain containing a free amino group, or a resin-bound polypeptide chain containing such an amino acid subunit, with a carbonyl-containing compound in the presence of sodium cyanoborohydride. Amino acids featuring a side chain containing a free amino group include Lys, ornithine, and diaminobutyric acid. The net effect of the reaction is to convert the carbonyl group



into a CH group bonded to the nitrogen of the sidechain free amino group of the resin-bound amino acid or polypeptide. For example, if the carbonyl compound is acetone



and the amino acid with the free amino-containing side chain is Lys (side chain = $-(CH_2)_3-CH_2NH_2$), the reaction produces an ε-N-isopropyl moiety. When the sodium cyanoborohydride reaction is complete, the modified peptide is cleaved from the resin support and purified by standard procedures.

The above-described synthesis can be used to prepare LHRH antagonists, as described below.

Example 1

Synthesis of The LHRH Antagonist

Ac-D-Nal-D-Phe-D-Phe-Ser-Tyr-D-Lys(iPr)-Phe-Lys (iPr)-Pro-Ala-NH_2

Referring to Fig. 3,
Ac-D-Nal-D-Phe-D-Phe-Ser(Bzl)-Tyr (Bzl)-D-Lys(FMOC)-Phe-D-Lys(FMOC)-Pro-D-Ala-benzhydrylamin e (0.25 mmole) resin was prepared by standard methods in a Beckman 990B automatic peptide synthesizer using 33% TFA for removal of the O-BOC protecting groups. The ε-FMOC protecting groups on the Lys residues are completely stable to these acidic conditions, and to subsequent neutralization steps with 10% triethylamine in chloroform. The resin was then treated with 50ml of a 50% solution of piperidine in DMF (dimethylformamide) for about 12h to remove the FMOC protecting group from the Lys residues.

To react the free ε-amino group of the Lys residues, the resin was mixed with 5ml of acetone, and 1 mmole of sodium cyanoborohydride in DMF/1% acetic acid added. The resin mixture was then stirred until it was negative to ninhydrin reaction (about 3h); the negative ninhydrin reaction indicated that the free ε-amino group had been converted to N-isopropyl amino groups.

The resin was then cleaved from the support by treatment with HF/anisole and purified under standard conditions to yield the desired polypeptide.

Ac-D-Nal-D-Phe-D-Phe-Ser-Tyr-D-Lys(iPr)-Phe-Arg-Pro-D-Ala-amide is prepared in analagous fashion using appropriate modifications of the above-described procedure.

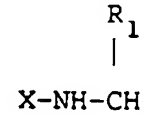
Use

The method of the invention can be used to modify any peptides of therapeutic or veterinary interest, e.g., hormones such as LHRH, TRH, and somatostatin, and analogs thereof. Such modifications can increase the chemical stability and potency of the peptides. In addition, the introduction of N-alkyl or aryl groups will minimize undesirable side effects, e.g., skin irritation, which are often present when the unalkylated or unarylated peptides are administered to human patients. Furthermore, the invention permits the addition of side groups (e.g., isopropyl) to amino acids in an inexpensive way, compared to methods in which the expensive pre-derivatized amino acid itself is employed.

Other embodiments are within the following claims.

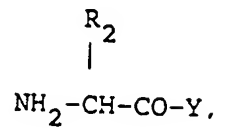
Claims

1. A method of solid-phase synthesis of a polypeptide having a non-peptide bond, comprising the steps of
   (a) providing an amino aldehyde of the formula

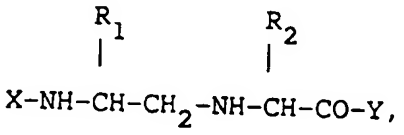


where X comprises a protecting group and R_1 is a side group of an amino acid,

(b) providing a complex of the formula

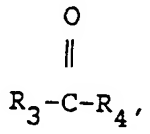


where Y comprises a solid phase and R_2 is a side group of an amino acid, and
(c) reacting said amino aldehyde with said complex in the presence of sodium cyanoborohydride to form

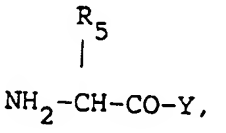


2. A method as claimed in claim 1 in which the said amino aldehyde is Trp aldehyde, Lys aldehyde or Cys aldehyde.

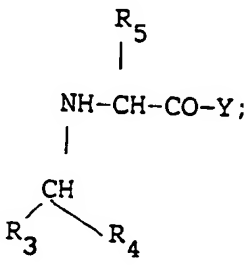
3. A method of solid-phase chemical modification of a peptide, comprising the steps of
a) providing a carbonyl-containing compound of the formula



where R_3 and R_4, independently, comprise hydrogen, branched or straight chain lower alkyl group, or aryl group;
(b) providing a complex of the formula

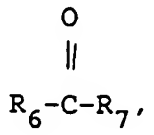


where Y comprises a solid phase and R_5 is a side group of an amino acid; and
(c) reacting said carbonyl-containing compound with said complex in the presence of sodium cyanoborohydride to form

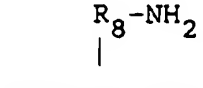


4. A method as claimed in claim 3 in which the said carbonyl-containing compound is formaldehyde.

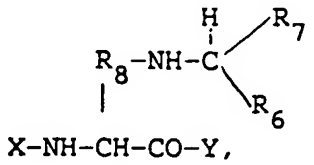
5. A method of solid-phase chemical modification of a peptide, comprising the steps of
(a) providing a carbonyl-containing compound of the formula



where R_6 and R_7, independently, comprise hydrogen, branched or straight chain lower alkyl group, or aryl group;
(b) providing a complex of the formula



where X comprises a protecting group, Y comprises a solid phase, and R_8-NH_2 is a side group of Lys, ornithine, or diaminobutyric acid;
(c) reacting said carbonyl-containing compound with said complex in the presence of sodium cyanoborohydride to form



(d) cleaving off said solid phase to release said peptide; and
(e) purifying said peptide.

6. A method as claimed in claim 5 in which the carbonyl-containing compound is acetone.

7. A method as claimed in claim 4 or claim 5 in which the R_8-NH_2 side group is a side group of Lys.

8. A method as claimed in any of claims 1 to 7 wherein Y represents either solely a solid phase or a solid phase to which one or more amino acids have already been bonded sequentially.

9. A method as claimed in any of claims 1 to 8 wherein X is a protecting group or a protecting group with one or two amino acids.

10. A method as claimed in any of claims 1 to 9 in which the solid phase is a resin.

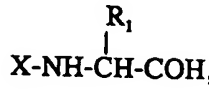
11. A method as claimed in claim 10 in which the resin is chloromethyl resin or benzhydrylamine-polystyrene resin.

12. A method as claimed in any of claims 1 to 11 further comprising the step of cyclizing the polypeptide.

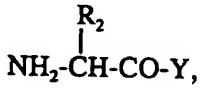
Patentansprüche

1. Verfahren zur Festphasen-Synthese eines Polypeptids, das eine nicht peptidartige Bindung enthält, umfassend folgende Schritte:

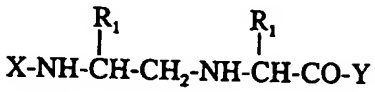
(a) Bereitstellung eines Aminoaldehyds der Formel



wobei X eine Schutzgruppe umfaßt und R_1 eine Seitengruppe einer Aminosäure ist;
(b) Bereitstellung eines Komplexes der Formel

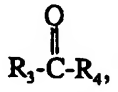


wobei Y eine Festphase umfaßt und R_2 eine Seitengruppe einer Aminosäure ist; und
(c) Reaktion zwischen dem Aminoaldehyd und dem Komplex in Gegenwart von Natriumcyanoborhydrid zur Bildung von

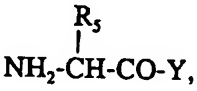


2. Verfahren nach Anspruch 1, wobei das Aminoaldehyd Trp-Aldehyd, Lys-Aldehyd oder Cys-Aldehyd ist.

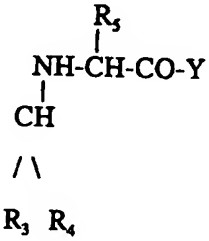
3. Verfahren zur chemischen Festphasen-Modifikation eines Peptids, umfassend folgende Schritte:
(a) Bereitstellung einer carbonylhältigen Verbindung der Formel



wobei R_3 und R_4 unabhängig voneinander Wasserstoff, eine verzweigt- oder geradkettige niedrige Alkylgruppe oder Arylgruppe sind;
(b) Bereitstellung eines Komplexes der Formel

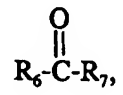


wobei Y eine Festphase umfaßt und R_5 eine Seitengruppe einer Aminosäure ist; und
(c) Reaktion zwischen der carbonylhältigen Verbindung und dem Komplex in Gegenwart von Natriumcyanoborhydrid zur Bildung von

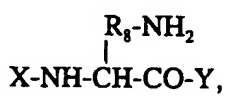


4. Verfahren nach Anspruch 3, wobei die carbonylhältige Verbindung Formaldehyd ist.

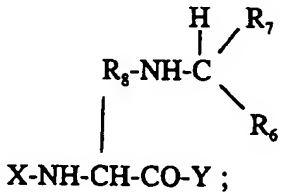
5. Verfahren zur chemischen Festphasen-Modifikation eines Peptids, umfassend folgende Schritte:
(a) Bereitstellung einer carbonylhältigen Verbindung der Formel



wobei R_6 und R_7 unabhängig voneinander Wasserstoff, eine verzweigt- oder geradkettige niedrige Alkylgruppe oder Arylgruppe sind;
(b) Bereitstellung eines Komplexes der Formel



wobei X eine Schutzgruppe umfaßt, Y eine Festphase umfaßt und R_8-NH_2 eine Seitengruppe von Lys, Ornithin oder Diaminobuttersäure ist;
(c) Reaktion zwischen der carbonylhältigen Verbindung und dem Komplex in Gegenwart von Natriumcyanoborhydrid zur Bildung von



(d) Abspalten der Festphase zur Freigabe des Peptids; und
(e) Reinigung des Peptids.

6. Verfahren nach Anspruch 5, wobei die carbonylhältige Verbindung Aceton ist.

7. Verfahren nach Anspruch 4 oder Anspruch 5, wobei die R_8-NH_2 Seitengruppe eine Seitengruppe von Lys ist.

8. Verfahren nach einem der Ansprüche 1 bis 7, worin Y entweder allein eine Festphase darstellt oder eine Festphase, an welche bereits eine oder mehrere Aminosäuren sequentiell gebunden sind.

9. Verfahren nach einem der Ansprüche 1 bis 8, worin X eine Schutzgruppe oder eine Schutzgruppe mit einer oder zwei Aminosäuren ist.

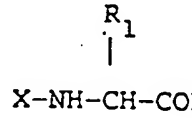
10. Verfahren nach einem der Ansprüche 1 bis 9, wobei die Festphase ein Harz ist.

11. Verfahren nach Anspruch 10, wobei das Harz Chlormethyl-Harz oder Benzhydrylamin-Polystyrol-Harz ist.

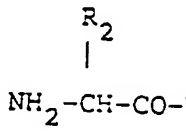
12. Verfahren nach einem der Ansprüche 1 bis 11, welches weiters den Schritt der Cyclisierung des Polypeptids umfaßt.

Revendications

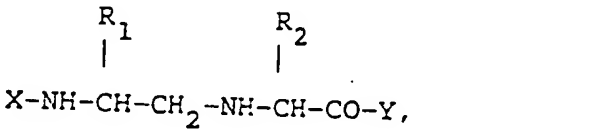
1. Procédé de synthèse en phase solide d'un polypeptide ayant une liaison non peptidique, comprenant les étapes de :
(a) mise en oeuvre d'un aminoaldéhyde de formule :



où X comprend un groupe protecteur et R_1 est une chaîne latérale d'un acide aminé,
(b) mise en oeuvre d'un complexe de formule :

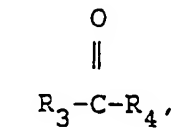


où Y comprend une phase solide et R_2 est une chaîne latérale d'un acide aminé, et
(c) réaction de l'aminoaldéhyde avec le complexe en présence de cyanoborohydrure de sodium pour former :

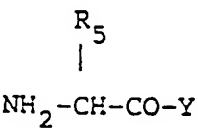


2. Procédé suivant la revendication 1, dans lequel l'aminoaldéhyde est l'aldéhyde Trp, l'aldéhyde Lys ou l'aldéhyde Cys.

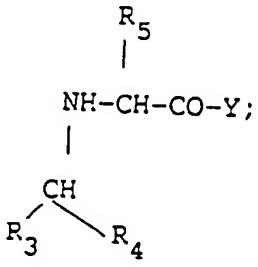
3. Procédé de modification chimique en phase solide d'un peptide, comprenant les étapes de :
(a) mise en oeuvre d'un composé contenant un carbonyle de formule :



où R_3 et R_4, indépendamment, comprennent hydrogène, radical alcoyle inférieur à chaîne branchée ou linéaire ou radical aryle,
(b) mise en oeuvre d'un complexe de formule :

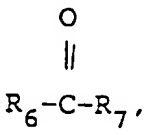


où Y comprend une phase solide et R_5 est une chaîne latérale d'acide aminé, et
(c) réaction du composé contenant un carbonyle avec le complexe en présence de cyanoborohydrure de sodium pour former :

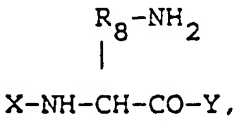


4. Procédé suivant la revendication 3, dans lequel le composé contenant un carbonyle est le formaldéhyde.

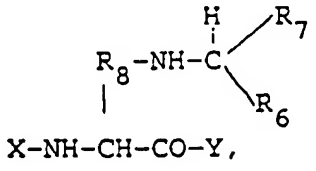
5. Procédé de modification chimique en phase solide d'un peptide, comprenant les étapes de :
(a) mise en oeuvre d'un composé contenant un carbonyle de formule :



où R_6 et R_7, indépendamment, comprennent hydrogène, radical alcoyle inférieur à chaîne branchée ou linéaire ou radical aryle,
(b) mise en oeuvre d'un complexe de formule :



où X comprend un groupe protecteur, Y comprend une phase solide et R_8-NH_2 est une chaîne latérale de Lys, ornithine ou acide diaminobutyrique,
(c) réaction du composé contenant un carbonyle avec le complexe en présence de cyanoborohydrure de sodium pour former :



(d) clivage de la phase solide pour libérer le peptide, et
(e) purification du peptide.

6. Procédé suivant la revendication 5, dans lequel le composé contenant un carbonyle est l'acétone.

7. Procédé suivant la revendication 4 ou 5, dans lequel la chaîne latérale R_8-NH_2 est une chaîne latérale de Lys.

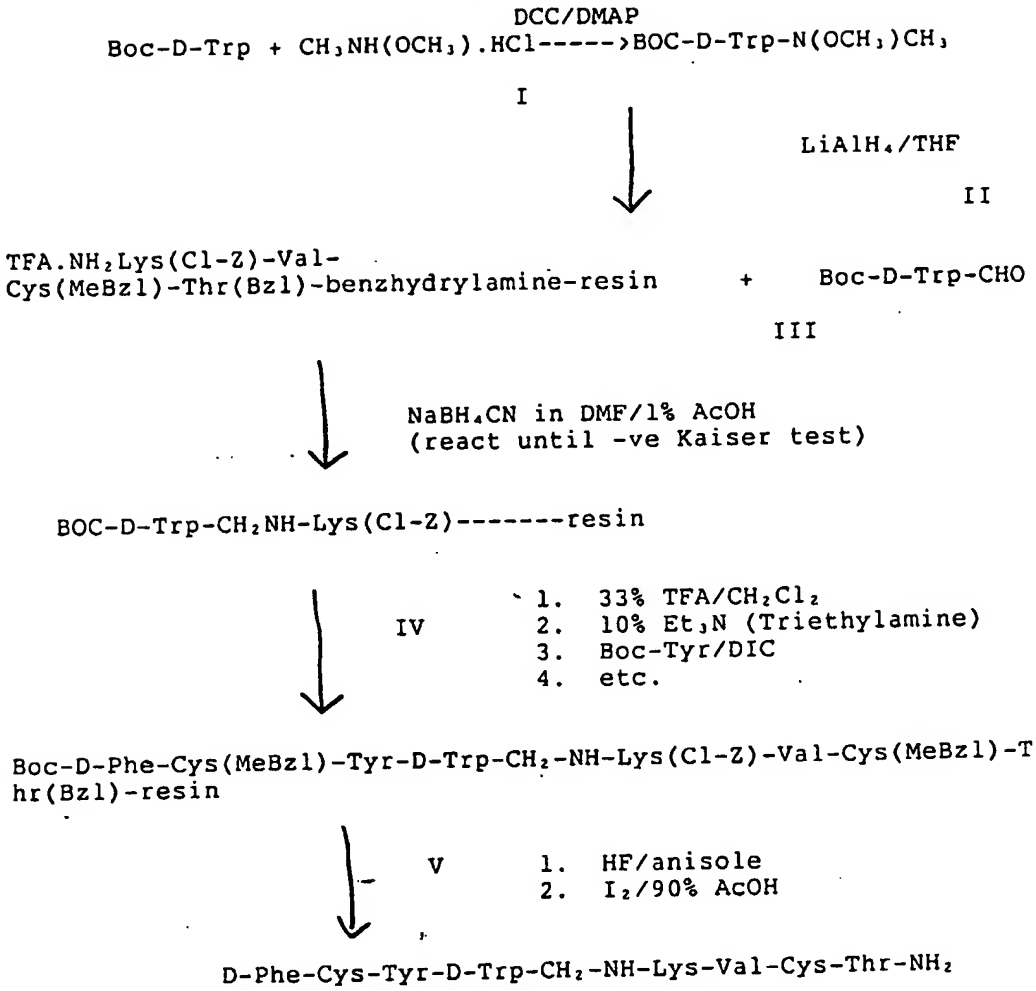
8. Procédé suivant l'une quelconque des revendications 1 à 7, où Y représente soit uniquement une phase solide, soit une phase solide à laquelle un ou plusieurs acides aminés ont déjà été fixés de manière séquentielle.

9. Procédé suivant l'une quelconque des revendications 1 à 8, où X est un groupe protecteur ou un groupe protecteur avec un ou deux acides aminés.

10. Procédé suivant l'une quelconque des revendications 1 à 9, dans lequel la phase solide est une résine.

11. Procédé suivant la revendication 10, dans lequel la résine est une résine chlorométhyle ou une résine benzhydrylamine-polystyrène.

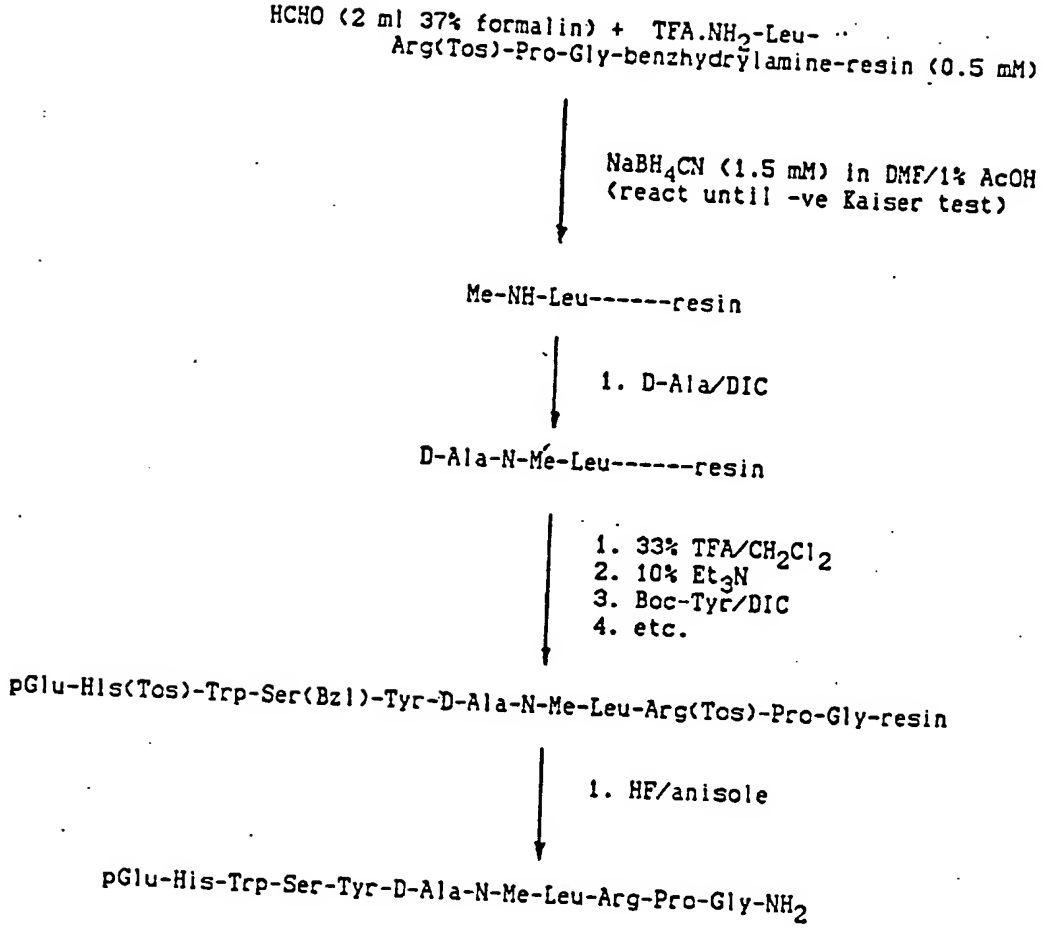
12. Procédé suivant l'une quelconque des revendications 1 à 11, comprenant de plus l'étape de cyclisation du peptide.



Abbreviations:

TFA:	trifluoroacetic acid
THF:	tetrahydrofuran
MeBzl:	methybenzyl
Bzl:	benzyl
Cl-Z:	4-chlorocabenzoxy
DMAP:	4-dimethyaminopyridine
DCC:	dicyclohexylcarbodiimide
DIC:	diisopropylcarbodiimide
HF:	hydrogenfluoride
I-V:	Reaction steps

Fig. 1



Abbreviations: TFA: trifluoroacetic acid
DIC: diisopropylcarbodiimide
HF: hydrogenfluoride
Tos: tosyl
Bzl: benzyl

Fig. 2

Ac-D-Nal-D-Phe-D-Phe-Ser(Bzl)-Tyr(Bzl)-D-Lys(FMOC)-Phe-Lys(FMOC)-Pro-D-Ala-benzhydrylamine resin

↓ 50% Piperidine/DMF, 12 h

Ac-D-Nal-D-Phe-D-Phe-Ser(Bzl)-Tyr(Bzl)-D-Lys-Phe-Lys-Pro-Ala-benzhydrylamine resin

↓ acetone/$NaCNBH_3$/DMF

Ac-D-Nal-D-Phe-D-Phe-Ser(Bzl)-Tyr(Bzl)-D-Lys(iPr)-Phe-Lys(iPr)-Pro-Ala-benzhydrylamine resin

↓ HF/anisole

Ac-D-Nal-D-Phe-D-Phe-Ser-Tyr-D-Lys(iPr)-Phe-Lys(iPr)-Pro-Ala-NH_2

Abbreviations: FMOC: fluorenylmethyloxycarbonyl
iPr: epsilon isopropyl
BZL: Benzyl

Fig. 3